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The carbon monoxide re-breathing method can underestimate Hbmass due to incomplete blood mixing

Keiser, Stefanie ; Siebenmann, Christoph ; Bonne, Thomas Christian ; Sørensen, Henrik ; Robach, Paul ; Lundby, Carsten

Abstract: **PURPOSE:** Hemoglobin mass (Hbmass) is commonly assessed using the CO re-breathing method with the subject in the seated position. This may lead to an underestimation of Hbmass as blood in lower extremity veins while seated may not be tagged with carbon monoxide (CO) during the re-breathing period. **METHODS:** To test this hypothesis, CO re-breathing was performed on four occasions in nine male subjects, twice in the seated position and twice in combination with light cycle ergometer exercise (1 W/kg body-weight) intending to accelerate blood circulation and thereby potentially allowing for a better distribution of CO throughout the circulation as compared to in the seated position. Blood samples were drawn from an antecubital vein and the saphenous magna vein following the re-breathing procedure. **RESULTS:** In the seated position, CO re-breathing increased the percent carboxyhemoglobin (%HbCO) in the antecubital vein to 8.9 % (7.8-10.7) [median (min-max)], but less ($P = 0.017$) in the saphenous magna vein [7.8 % (5.0-9.9)]. With exercise, no differences in %HbCO were observed between sampling sites. As a result, CO re-breathing in combination with exercise revealed a 3 % higher ($P = 0.008$) Hbmass, i.e., 936 g (757-1,018) as compared to 908 g (718-940) at seated rest. **CONCLUSION:** This study suggests an uneven distribution of CO in the circulation if the CO re-breathing procedure is performed at rest in the seated position and therefore can underestimate Hbmass.

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The carbon monoxid re-breathing method **can underestimate Hb_{mass} due to incomplete blood mixing**

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Abstract

Purpose: Hemoglobin mass (Hb_{mass}) is commonly assessed using the CO re-breathing method with the subject in the seated position. This may lead to an underestimate of Hb_{mass} as blood pooled in lower extremity veins while seated may not be tagged with CO during the re-breathing period.

Methods: To test this hypothesis, CO re-breathing was performed on four occasions in nine male subjects, twice in the seated position and twice in combination with light cycle ergometer exercise (75 W) intending to accelerate blood circulation and thereby potentially allowing for a better distribution of CO throughout the circulation as compared to in the seated position. Blood samples were drawn from an antecubital vein and the saphenous magna vein following the re-breathing procedure.

Results: In the seated position CO re-breathing increased the percent carboxyhemoglobin (%HbCO) in the antecubital vein to 8.9 % (7.8 - 10.7) (median (min – max)), but less ($P=0.017$) in the saphenous magna vein (7.8 % (5.0 - 9.9)). With exercise no differences in %HbCO were observed between sampling sites. As a result CO re-breathing in combination with exercise revealed a ~3% higher ($P = 0.008$) Hb_{mass} , i.e. 936 g (757 - 1018) as compared to 908 g (718 - 940) at seated rest.

Conclusion: This study suggests an uneven distribution of CO in the circulation if the CO re-breathing procedure is performed at rest in the seated position and therefore an underestimate of Hb_{mass} .

Key words

nHb, red cell mass, blood volume, CO, RCV

Introduction

Methods that quantify hemoglobin mass (Hb_{mass}) or red blood cell volume (RCV) in humans are based on the dilution principle. A given quantity of tracer is administered to the circulation, and the subsequent concentration in the blood allows for calculation of Hb_{mass} or RCV. The use of radioactive tracers such as ^{51}Cr is considered the gold standard (Haematology 1980). However, also other tracers such as carbon monoxide (CO), are in common use. The low technical requirements and smaller ethical concerns associated with the CO re-breathing method may have made this procedure popular. Furthermore, as the CO re-breathing method is associated with a typical measurement error similar to the ^{51}Cr method (Gore et al. 2005) it is considered as valid and reliable (Ashenden et al. 1999; Burge and Skinner 1995; Hutler et al. 2000; Thomsen et al. 1991). A prerequisite for any dilution principle and thus also the CO re-breathing method is a uniform distribution of the tracer, i.e. the pulmonary administrated CO, over the circulation. Since there is no transfer of CO between hemoglobin molecules (Blackmore 1970), only red blood cells passing the pulmonary circulation may be tagged with CO. However, the CO re-breathing procedure is commonly conducted at seated rest (Lundby et al. 2007; Morkeberg et al. 2011; Prommer et al. 2008; Robach et al. 2006; Schmidt and Prommer 2005; Steiner and Wehrlin 2011; Ulrich et al. 2011) or semi recumbent rest (Garvican et al. 2010). In the supine position total blood turnover in large pooling veins may take up to 30 min (Wennesland et al. 1962), and likely even longer in the seated position as gravitation promotes blood accumulation in the leg veins. If erythrocytes remain pooled in lower extremities during a CO re-breathing procedure they cannot be tagged with CO which may lead to an underestimation of Hb_{mass} . Complete blood mixing has previously been suggested to occur within 6 to 10 min based on similar %HbCO values in blood samples obtained from the radial artery, earlobe capillaries and an antecubital vein (Garvican et al. 2010; Prommer and Schmidt 2007). These studies however all failed to include blood sampling from large pooling veins of the lower body where turnover is known to be much slower.

We further speculated that a potential non-uniform distribution of CO may be prevented if the CO re-breathing procedure is performed in combination with light exercise. Exercise accelerates blood circulation and mobilizes venous blood through the muscle pump effect. This may facilitate blood pooled in the lower extremities to pass the pulmonary circulation and accordingly be tagged with CO.

In summary we hypothesized that i) CO re-breathing performed in the resting seated position will lead to higher %HbCO values in an antecubital vein than in the saphena magna vein of the lower leg, ii) exercise abolishes this difference by enhancing blood circulation and, as a consequence, iii)

the implementation of light exercise to the CO re-breathing procedure reveals a higher Hb_{mass} suggesting that CO re-breathing performed in the resting seated position underestimates Hb_{mass} .

Methods

This study was approved by the Research Ethics Committee of ETH Zurich, Switzerland (EK 2012-N-07) and the experimental protocols conformed to the Declaration of Helsinki.

Subjects

Nine healthy men (26.7 ± 3.8 yr, 1.81 ± 0.08 m, 75.6 ± 6.0 kg, mean \pm SD) were included as subjects. All were recreational physical active but not involved in organized exercise training at a high level. All subjects gave oral and written informed consent before participation. Subjects were excluded from the study if they had donated blood within the last three months prior to the study and were not allowed to donate blood before the study was completed.

Study design

Subjects reported to the laboratory on four consecutive days. They performed two CO re-breathing maneuvers at rest in the seated position and two in combination with mild exercise (see below). To exclude a cumulative effect of administered CO all measurements were separated by at least 24 h, which is sufficient as judged by the pre %HbCO values. The first measurement was always carried out at rest to allow for familiarization without the stress of exercise. The order of the following three measurements was randomized.

Protocol

Resting seated CO re-breathing. The seated CO re-breathing was performed in a slightly modified version of the (Burge and Skinner 1995) protocol. The re-breathing system was checked for leaks by water submersion prior to use. A 20-G catheter was placed in an antecubital vein and in the *v. saphena magna* at the ankle. The distance from the sampling side to the heart was about 30-40 cm and 90-110 cm respectively. After placement of the catheter the stasis was removed and care was taken not to apply any pressure on the subject in order not to limit venous blood flow. The subjects then rested in the seated position for 20 min with the arms hanging loosely down. During this time they ingested 500 ml of water to stabilize plasma volume. The subjects thereafter breathed 100% O₂ for 4 min through an open circuit system (100 l Douglas bag) to eliminate nitrogen from the

airways. Thereafter, a 2 ml blood sample was drawn from the antecubital vein and analyzed in quadruplicate for %HbCO and Hb concentration ([Hb]) on a hemoximeter (ABL800, Radiometer, Copenhagen, Denmark) and for hematocrit (Hct) by the micro method (4 minutes at 13,500 rpm). The open circuit was then switched to a closed re-breathing circuit (previously flushed and filled with O₂) with an integrated CO₂-absorber containing 1 kg of soda lime and 4 l re-breathing bag. After a few breaths a 200 ml syringe with 1.5 ml/kg body-mass of 99.997 % chemically pure CO (CO N47, Air Liquide, Pullach, Germany) was administrated to the circuit and re-breathed for 10 minutes. At the end of the re-breathing period a second blood sample was obtained from the antecubital vein. Simultaneously, a further blood sample was obtained from the saphena magna vein (only after the first resting seated and exercise protocols, respectively, whereas the saphena magna vein was not punctured in the other two measurements). Both samples were analyzed as the initial blood sample.

Since CO clearance from the circulation is increased with hours of elevated pulmonary ventilation (Bruce and Bruce 2006) we tested the potential bias (increased Hb_{mass}) introduced to the measurement by the increased VE during exercise. It should be acknowledged however that our measurements are completed with the subjects connected to a closed system, and hence any CO potentially cleared by a higher ventilation will be re-breath. The proof-of-concept validation was done in four separate subjects not taking part in other parts of the experiments. The above described CO re-breathing procedure was performed on two separate days in these subjects 1) with normal voluntarily VE and 2) with the subjects hyperventilating at ≈ 30 l/min (measured with a Quark b2, Cosmed, Italy). HbCO values in the blood samples obtained from the antecubital vein after 10 minutes of CO re-breathing were as follows: Subject 1: 7.3 vs 7.2%; subject 2: 8.2 vs 8.4%; subject 3: 9.7 vs 9.9% and for subject 4: 8.1 vs 7.9% for measurements completed on Day 1 vs Day 2, respectively. Hence the increased VE during exercise does not seem to affect our measurements.

Exercise CO re-breathing. The CO re-breathing procedures performed during exercise was carried out as in the seated position for all main points. After completing the same 20 min of rest and the consumption of 500 ml of water, the subjects were moved from the chair to a cycle ergometer (Monark Ergomedic 839 E, Sweden). The 4 min of 100% O₂ breathing was initiated simultaneously with the onset of light exercise (75W). The subjects continued to exercise throughout the entire re-breathing protocol and blood sampling. At the end of the 4 min period a blood sample was obtained from the antecubital vein and subjects were switched to the closed circuit. After a short familiarization period an identical amount of CO as in the resting seated protocol was administrated and re-breathed for 10 min. At the end of the re-breathing period blood samples were drawn

simultaneously from the antecubital vein and from the saphena magna vein (only after the first exercise measurement). All CO re-breathing procedures were performed by the same operator.

Calculations

The mean difference in %HbCO between the first and the second blood sample was used to calculate Hb_{mass}. Red blood cell volume (RCV), plasma volume (PV) and blood volume (BV) were then derived by using Hb_{mass}, [Hb] and Hct. The blood parameters were calculated using standard equations (Burge and Skinner 1995).

Values from day one from the seated and the exercise protocol were used to calculate the difference between the antecubital vein and the v. saphena magna. The second trial of both methods was used for TE and Hb_{mass} calculations. The latter values represent the mean value from the two days of each protocol.

Statistics

Statistical evaluation of the data was performed by using the software IBM SPSS 20. Systematic errors could not be found. As the data was not normally distributed, nonparametric Kolmogorov-Smirnov tests were used to compare the medians. A p-value < 0.05 was considered statistically significant. The coefficient of variation for the Hb_{mass} for the two testing models was expressed as the typical error, which is calculated as the standard deviation of difference scores divided by $\sqrt{2}$ (Hopkins 2000). When expressed as a percent of the mean, the percent typical error (%TE) is obtained. The results are presented as median (min - max).

Results

Antecubital vs saphena magna vein %HbCO

Values for %HbCO are shown in Table 1. It was not possible to puncture the saphena magna vein in one subject. Hence, for this specific analysis 8 instead of 9 subjects were included. The median difference in %HbCO between the antecubital and saphena magna vein sample was 0.55 (0.20 - 5.68) % in the resting seated position, with the value being higher in the antecubital samples in all subjects (Figure 1). With exercise the difference in %HbCO was 0.01 (-0.30 - 0.20) % ($P = 0.017$ vs. resting protocol). This indicates incomplete blood mixing during the CO re-breathing procedure performed in the seated position but not during the exercise protocol.

The effect of exercise (vs rest) CO re-breathing on Hb_{mass} values

Values for Hb_{mass} , [Hb], Hct, and blood volume parameters are presented in Table 2. We observed an increase in most blood volume variables when the CO re-breathing was performed during exercise. Figure 2 illustrates the Hb_{mass} yielded by the two protocols. The median Hb_{mass} increased ($P = 0.008$) from 908 (718 - 940) g measured in the seated position to 936 (757 - 1018) g obtained with the exercise protocol. The increase in Hb_{mass} with exercise was a uniform finding across all subjects.

Reliability of the two methods

The TE was 1.7% for the resting seated protocol and 2.4 % for the exercise protocol. No statistical difference was found ($P = 0.515$) when comparing the median scores of each method between the first and the second day.

Discussion

This study indicates an uneven distribution of CO in the blood circulation if the CO re-breathing procedure is performed in the resting seated position. In accordance with our hypothesis we observed higher %HbCO in the antecubital than in the saphenous magna vein following CO re-breathing performed in the resting seated position whereas this difference was abolished when re-breathing was performed during light exercise.

The increase in saphenous venous %HbCO in the exercise protocol is likely accounted for by a mobilization of blood pooled in the veins of the lower extremities leading to more complete blood mixing. Complete blood mixing has previously been suggested to occur within 6 to 10 min based on similar %HbCO values in blood samples obtained from the radial artery, earlobe capillaries and an antecubital vein (Garvican et al. 2010; Prommer and Schmidt 2007). These studies however all failed to include blood sampling from large pooling veins of the lower body, and this may explain the difference in results. In contrast, our data indicates that complete blood mixing does not occur within a 10 min period, as also suggested previously (Wennesland et al. 1962).

In addition to enhanced circulation, splenic contraction during exercise might be an additional explanation for the higher Hb_{mass} values obtained during the exercise trial. In humans ~10 % of Hb_{mass} is stored in the spleen in resting conditions (Stewart and McKenzie 2002). With exercise a relative decrease in splenic erythrocyte content is a common observation and may reach 40-60% at maximal workloads (Flamm et al. 1990; Froelich et al. 1988; Laub et al. 1993; Sandler et al. 1984). With light exercise similar to that in the present study, a reduction in splenic blood content of about 10% has been reported against seated rest (Laub et al. 1993). Assuming a splenic volume of no more than 300 ml this would result in a 30 ml increase in circulating whole blood. Assuming a splenic Hct of 80%, a 10% reduction in splenic blood content will correspond to 25 ml of red cells entering the circulation. Since we observed an increase of 85 ml in RCV in the exercise trial the contribution of splenic contraction to the observed increase in Hb_{mass} with exercise may account for up to 28%. It should be noted that the typical error for the measurements increased (not statistically significant) from 1.7 to 2.4 when using the exercise protocol. Both values are however within the expected values for the method (Gore et al. 2005). The source for the increased TE with exercise remains unknown. One limitation of the exercise protocol is the unknown amount of CO that may have remained in the re-breathing circuit after each procedure. We made the assumption (Thomsen et al. 1991) that 2.2% of the CO remains within the re-breathing circuit and that this is similar for both protocols. However, since exercise accelerates blood circulation and ventilation, the absorbed CO during the 10 minutes of re-breathing may have been higher during the exercise protocol. However, an underestimation of absorbed CO from the re-breathing circuit would have

resulted in an underestimation of Hb_{mass} in the exercise trial and further support our conclusions. Another potential limitation to the exercise (and seated) protocol is the unknown amount of CO that may leave the vascular space and subsequently bind to myoglobin. Based on theoretical reasoning, CO loss from the circulation is estimated to be 1.6 ml CO in ten minutes at rest (Garvican et al. 2010). During exercise, more muscle capillaries are opened which may facilitate CO diffusion into the myocytes, but this remains speculative. A further limitation to our study is that we did not obtain %HbCO in the saphena magna vein before the re-breathing of CO, but assumed this value to be equal to %HbCO in the antecubital vein sample. It seems however very unlikely that differences between those two sampling sites in terms of %HbCO should exist in the resting human.

In conclusion, a CO re-breathing procedure performed in the resting seated position may underestimate Hb_{mass} due to uneven distribution of CO over the circulation, and we recommend adding light exercise to the CO re-breathing protocol. Future studies may attempt to decrease the re-breathing period if the CO is administered in combination with light exercise. Due to the higher blood flow in the pulmonary circulation re-breathing shorter periods may prove sufficient which would make the re-breathing procedure when combined with exercise easier to conduct. As an alternative to exercise it may be favorable to perform the CO re-breathing procedure in the complete supine position and perhaps in combination with passive movement of the extremities. Supine rest could also be combined with slight head-up and down tilts to facilitate movement of central and peripheral blood volumes. Since strenuous exercise prior to the measurement of Hb_{mass} has been shown not to influence the results (Robach et al. 2012) or to spuriously increase the values (Gough et al. 2012) it at present remains unknown if exercise prior to the measurement may be sufficient to facilitate a better CO distribution within the circulation.

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TABLE 1. Antecubital (AC) and saphenous magna (SM) venous carboxyhemoglobin values (%HbCO) in all subjects after 10 minutes of CO re-breathing with seated rest (seated) or light ergometer exercise (exercise). * indicates statistical differences between the measurements ($P < 0.05$).

subject	seated 1			seated 2		exercise 1			exercise 2	
	Δ AC					Δ AC				
	AC	SM	SM	AC	SM	AC	SM	SM	AC	SM
1	8.85	7.30	1.55	9.25	-	8.45	8.60	-0.15	8.45	-
2	9.9	9.70	0.20	10.30	-	9.25	9.10	0.15	9.75	-
3	9.55	9.33	0.23	9.45	-	8.65	8.95	-0.30	8.78	-
4	10.70	5.03	5.68	10.98	-	10.00	9.98	0.03	10.08	-
5	9.10	5.03	4.08	8.65	-	8.50	8.40	0.10	8.65	-
6	8.23	8.00	0.23	8.45	-	7.30	7.45	-0.15	7.88	-
7	7.80	7.38	0.43	7.90	-	7.18	7.18	0.00	7.83	-
8	8.90	8.23	0.68	9.10	-	8.60	8.40	0.20	8.33	-
median	9.00	7.69	0.55*	9.18	-	8.55	8.50	0.01*	8.55	-

TABLE 2. Total hemoglobin mass (Hb_{mass}) and blood volume parameters for both CO re-breathing protocols. Values are medians (minimum-maximum).

	seated CO re-breathing	exercise CO re-breathing	P-values
	n = 9	n = 9	
Hb_{mass} (g)	908 (718-940)	936 (757-1018)	0.008
Hb_{mass}/kg (g/kg)	11.4 (10.3-13.3)	12.0 (10.8-14.3)	0.008
[Hb] (g/dl)	14.9 (13.5-15.9)	15.3 (14.2-16.2)	0.051
Hct (%)	43.8 (40.1-46.6)	45.9 (42.1-47.5)	0.050
RCV (ml)	2670 (2089-2841)	2862 (2165-3024)	0.008
PV (ml)	3408 (2436-4180)	3255 (2802-4155)	0.374
BV (ml)	6094 (4526-6981)	6065 (4971-7179)	0.374

Figure legends

Figure 1. Difference in carboxyhemoglobin (%HbCO) in blood samples drawn from a antecubital and the saphena magna vein during the CO re-breathing procedure performed in the resting seated position and during light exercise. In the Box-Plots the dashed lines indicate the mean and the solid lines the median scores. The white points illustrates the individual differences in HbCO of every subject ($n = 8$). The difference in %HbCO between antecubital and saphena magna at rest varies greatly between subjects and may be related to vessel structure, degree of sympathetic constraint or different degrees of constraints to venous blood flow while seated.

Figure 2. Illustration of the total hemoglobin mass (Hb_{mass}) obtained during the resting seated position and with light cycle-ergometer exercise when using anticubital vein blood samples. In the Box-Plots the dashed lines indicate the mean and the solid lines the median scores. The white points illustrates the individual Hb_{mass} of every subject ($n = 9$).

Figure 1

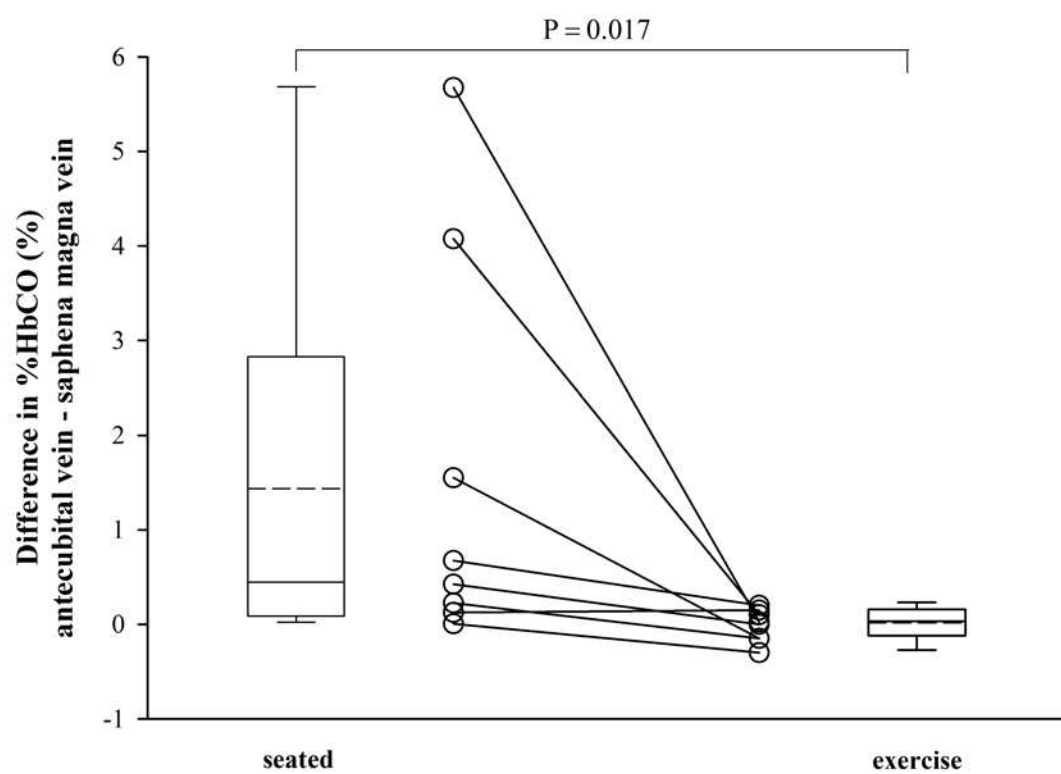


Figure 2

